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Identification and comparative analysis of immune-related genes and signaling pathways in the silkworm, *Bombyx mori*

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Abstract: The silkworm, *Bombyx mori*, has been a domesticated, economically important insect for 5 000 years. Recent accomplishments in molecular immunology have revealed just a preliminary outline for silkworm innate immunity. The acquisition of the updated silkworm genome has enabled a comparative analysis of the silkworm immune-related genes and signaling pathways. In this study, through comparing with the sequenced *Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera* and *Tribolium castaneum* genomes, we identified over 218 genes in the silkworm that fall into 21 families involved in immune defense, including pattern recognition receptors, signaling transducers, effectors and oxidative defense enzymes. Phylogenetic analysis showed that the signal transducers have remarkable orthologous relationships between different insect species in spite of the divergent sequences. In contrast, gene families associated with recognition, modulation and effectors exhibit more significant sequence conservation. However, the orthologs of these families are remarkably absent, presumably attributable to the lineage-specific gene duplication. Our results suggest that common mechanisms may be responsible for innate-immunity responses to pathogens via signaling pathways in the silkworm. Furthermore, hosts may adjust their defensive strategies by gene duplication and sequence divergence to kill pathogens.

Key words: *Bombyx mori*; innate immunity; antimicrobial peptide; oxidative defense; gene expression; signaling pathways

1 INTRODUCTION

The silkworm, *Bombyx mori*, is an economically important insect domesticated for over 5 000 years. Studies on the silkworm diseases have made a great contribution to modern insect pathology since the 19th century such as the diseases caused by *Beauveria bassiana* or *Nosema bombycid* infections. Many findings in silkworm pathology have promoted studies on insect immunity. Since the 1980s, studies have focused on the molecular basis of immunity in the silkworm. Immune-related proteins such as antimicrobial peptides (AMPs) and pattern recognition receptors (PRRs) have been isolated and purified from hemolymph of the silkworm larvae (Morishima *et al.*, 1990; Yoshida *et al.*, 1996).

Innate immunity is the first line of molecular defense against foreign invaders in invertebrates and vertebrates with the exception of physical barriers. Insect immunity shares the immune pathway and many components with vertebrates, including a diverse set of reactions such as the recognition and activation of NF-κB transcription factors, and the synthesis of AMPs. Functional researches have made progress in *Drosophila* molecular immune mechanisms, which promote the research on insect immune defense (Lemaitre and Hoffmann, 2007). Comparative genomics was performed on complete genome sequence, and the evolutionary model of immune-related genes and pathways in Diptera was proposed (Christophides *et al.*, 2002; Sackton *et al*., 2007; Waterhouse *et al.*, 2007). Species-specific gene duplication was obvious in *Drosophila melanogaster* and *Anopheles gambiae*. However, only six genes were defined from four gene families encoding AMPs in *Apis mellifera*, a significantly smaller number than in *D. melanogaster*. The honey

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bees possess only one-third of the number of the insect immunity genes, suggesting that the social insects might resist infection using group strategies (Evans *et al.*, 2006). In the evolutionary tree, the silkworm (Lepidoptera) is located between the honey bee (Hymenoptera) and the mosquito (Diptera) (Zdobnov and Bork, 2007). These comparative and evolutionary analyses of immune-related genes have revealed the conserved mechanisms of immune defense in different insects.

Immune-related genes against bacteria, fungi and parasites in *B. mori* have also been well characterized. In particular, many molecules have been isolated and purified from *B. mori*, including AMPs and PRRs. The first insect receptor that binds with peptidoglycans was identified from *B. mori* and named peptidoglycan recognition receptor (PGRP) (Yoshida *et al.*, 1996). Two REL/NF-κB like transcription factors, Dorsal and Relish homologs, have been identified in *B. mori*. Interestingly, they activate the expression of AMP genes by a unique gene-regulation model (Tanaka *et al.*, 2005; Tanaka *et al.*, 2007). Spatzle (Spz), a ligand of Toll receptor, can enhance the expression of AMP genes (Wang *et al.*, 2007). Moreover, in the lepidopteran *Manduca sexta*, the prophenoloxidase (PPO) activation and serine protease cascades have been well defined in nodule formation, melanotic encapsulation and wound healing (Kanost *et al.*, 2004). These results suggest that Lepidoptera possess the conserved signaling pathways like Diptera.

On the basis of the 6x-coverage silkworm genome, we have identified some immune-related genes, primarily including genes encoding AMPs (Xia *et al.*, 2004; Cheng *et al.*, 2006). Furthermore, the acquisition of the updated silkworm genome assembled by Chinese and Japanese scientists has provided an opportunity to perform a comparative analysis of the immune-related genes. In this paper, we (i) identified the likely homologs involved in the immune defense, including recognition, signaling transduction and effectors, (ii) located the genes on chromosome markers of single nucleotide polymorphism (SNP) and (iii) compared the evolutionary relationship of immune-related genes in the dipteran and hymenopteran genomes.

Abbreviations: AMP: antimicrobial peptide; PAMP: pathogen-associated molecular pattern; PRR: pattern recognition receptor; PGRP: peptidoglycan recognition receptor; GNBP: Gram-negative bacteria-binding protein; SCR: scavenger receptor; CRD: carbohydrate recognition domain; PPO: prophenoloxidase; PAP: PPO-activating protease; LPS: lipopolysaccharide; ROS: reactive oxygen species; *Bm*: *Bombyx mori*; *Dm*: *Drosophila melanogaster*; *Ag*: *Anopheles gambiae*; *Am*: *Apis mellifera*.

2 MATERIALS AND METHODS

2.1 Identification of immune-related genes in *B. mori*

Sequences of immune-related genes in *D. melanogaster* and *A. gambiae* were downloaded from an insect immunology database (http://cegg.unige.ch/insecta/immunodb) and were used as queries to search for immune-related genes in the updated assembled silkworm genome sequence by the International Silkworm Genome Sequencing Consortium (2007). An E-value threshold of 10^{-6} was set to select candidate sequences when local TBLASTN search were carried out. To predict the exon/intron boundaries, the corresponding genomic sequences were annotated by the FGENESH program (http://sun1.softberry.com/berry.phtml). The resultant similar sequences were then manually confirmed against the non-redundant protein sequence (nr) database.

2.2 Chromosomal location

The chromosomal locations for immune-related genes were identified in the silkworm database (http://silkworm.swu.edu.cn/silkdb/) and the supplemental figures using the single nucleotide polymorphism markers (Yamamoto *et al.*, 2006).

2.3 Phylogenetic analysis

Multiple sequence alignments of each family were performed using "muscle", a public domain protein multiple sequence alignment software (http://phylogenomics.berkeley.edu/cgi-bin/muscle/input_muscle.py). Phylogenetic trees were reconstructed from these alignments by the neighbor-joining algorithm implemented in ClustalW with options of 1 000 bootstrap sample (Thompson *et al.*, 1994). Confident orthologous relationships were assigned if the relevant bootstrap support was greater than 60%. To compute *Bm-Dm* and *Bm-Am* orthologous distances, confidently aligned conserved regions were extracted using Gblocks, excluding all gap positions (Castresana, 2000). The sequence relationships among these conserved protein cores were estimated in terms of amino acid substitutions using the maximum likelihood (ML) algorithm implemented with PhyML (Guindon and Gascuel, 2003), which takes into account the different substitution rates among lineages and sites.

2.4 ESTs collection

To validate the expression of immunity-related genes, a BLASTN search was performed against the silkworm EST database SilkDB (http://silkworm.swu.edu.cn/silkdb/) and Silkbase (http://morus.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). Assembled exon sequences of the immunity-related genes were used as queries for the search. The putative ESTs were identified based on sequence similarity with the query. Ninety percent or greater identity was considered to be a potential EST corresponding to a specific gene.

3 RESULTS

3.1 Immunity-related genes and pathway

Using large-scale analyses on four insect species (Diptera, Hymenoptera, Coleoptera and Lepidoptera), we have identified several genes from the silkworm genome that may be involved in immune response. In total, 218 genes were characterized from 21 immune-related families or superfamilies including pattern recognition, signaling modulation and effectors (Table 1). About 65% of the genes are expressed; about 90% of the genes were mapped to specific chromosomes. Phylogenetic analyses suggest that most gene families have experienced lineage-specific duplication or gene losses that may reflect specific evolutionary pressures. We identified 45 genes forming 1∶1∶1∶1 orthologs (*Bm/Dm/Ag/Am*). The core members of the four pathways were analyzed and placed into 1∶1∶1∶1 orthologs in four insect species by phylogenetic analysis, and they may play similar functions in these insects. The remaining genes show relationships that derive from family duplications and gene loss, or correspond to cases where confident determination of phylogenetic relationships is precluded due to high sequence divergence.

Table 1 The number of immune-related genes in four insect species

Gene family	*Bomyx mori*	*Drosophila melanogaster*	*Anopheles gambiae*	*Apis mellifera*	*Tribolium castaneum*
Recognition					
PGRP	11	13	7	4	7
GNBP	4	3	7	2	3
Galectin	2	6	10	2	3
C-type lectin	22	34	25	10	16
Fibrinogen-domain	3	14	61	2	7
Scavenger	12	22	19	14	21
Signalling					
Spz	6	6	6	2	7
CLIP-SP	15	45	56	18	48
Serpin	15	30	17	7	31
Toll	13	9	10	5	9
REL	2	3	2	4	4
Components#	17	18	18	18	25
Effectors					
Prophenoloxidase	3	3	9	1	3
TEP	6	6	13	4	4
LYS	3	13	8	3	4
AMP*	39	21	10	6	12
Others					
Caspase	4	7	14	4	7
Catalase	7	2	1	5	4
Inhibitors of apoptosis	5	4	8	7	4
Peroxidase	23	20	26	17	20
Superoxide dismutatse	6	4	5	4	4
Total	218	283	332	139	243

#: The components from immune pathways including Toll, Imd, JNK, and JAKSTAT pathway. *: Cecropins, moricins, gloverins, attacins, enbocins, lebocins, defensin, diptericins, drosomycins, drosocin, metchnikowin, gambicin, holotricin, abaecin, apidaecin, apisimin, and hymenoptaecin.

3.2 Gene duplication and divergence

Compared to the *D. melanogaster* and *A. mellifera* genomes, *B. mori* possesses roughly the same number of immune-related genes as *D. melanogaster* but has a higher number of genes than *A. mellifera*. Duplications of immune genes have resulted in species-specific duplications and generation of novel genes in the silkworm and Dipteran genomes. Examples of species-specific duplication are AMP genes, such as cecropin and moricin families (Fig. 1). The moricin gene cluster family has 12 members located on chromosome 23. Based on the high similarity in nucleotide sequence, eight genes of the moricin B sub-family appear to be produced by several sequential duplication events about 1.9 MYA ago (Cheng *et al.*, 2006). Another species-specific gene duplication is evident in the cecropin family comprised of cecropins and enbocins. With the exception of the cecropin A sub-family, the rest of the genes including the enbocins cluster are located on chromosome 26. In *D. melanogaster*, the drosomycin family includes seven members with diverse sequences and structures, resulting in the functional divergence for antifungal activities (Yang *et al.*, 2006). However, such functional expression is not evident in the AMP gene of *A. mellifera*. PGRP family has two main clusters located on chromosome 1 (PGRP1 - 5) and 16 (PGRP8 - 11). Eight Toll-related genes were found to cluster with wide gaps for each other on chromosome 23 (Cheng *et al.*, 2007). Interestingly, no duplication was observed for several families, including PPO, lysozyme (LYS), fibrinogen-related protein (FREP) and thioester-containing protein (TEP). These families have roughly the same number of genes as the honey bee. However, they appear to be species-specifically expanded in Diptera. For example, *D. melanogaster* has 13 LYSs and *A. Gambia* has 9 PPOs, 13 TEPs and 61 FREPs, suggesting that these gene families in Diptera underwent greater selective pressures compared with Lepidoptera and Hymenoptera (Waterhouse *et al.*, 2007).

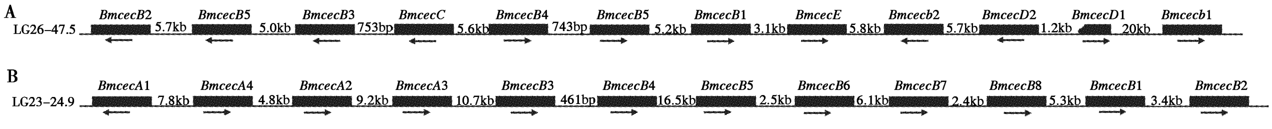

Fig. 1 Two gene clusters of AMP genes of *Bombyx mori*

A: Cecropin family located on chromosome 26; B: Moricin family located on chromosome 23. Black arrowheads indicate the orientation of gene transcription.

It has been observed that immunity genes are significantly more divergent compared to the other genes in Dipteran genomes (Waterhouse *et al.*, 2007). These results suggest that genes encoding recognition receptors and effectors evolve under positive Darwinian selection (Sackton *et al.*, 2007). To compare the divergence distance of immune-related genes of *B. mori* with *D. melanogaster* and *A. mellifera*, we calculated the distance of the identified orthologous genes (*Bm/Dm/Am*). A similar measurement was used for some immune-related gene orthologs of *Bm-Dm* and *Bm-Am*. The results indicate that the immune gene families have differences in sequence conservation. A plot of *Bm-Dm* and *Bm-Am* phylogenetic distances suggest that, on average, signaling orthologs are significantly more divergent than those genes involved in immune recognition and modulation (Fig. 2). Interestingly, the average distance of *Bm-Am* signaling orthologs (1.44) is shorter than that of *Bm-Dm* (1.62), suggesting that the signal transducers of the silkworm are closer to those of *A. mellifera*.

3.3 Immune effectors

AMPs have low molecular weight (< 10 kDa) and broad-spectrum antimicrobial activities against bacteria and/or fungi. In general, they are expressed in fat bodies in response to infection. Genes encoding AMPs are highly diverse. In *D. melanogaster*, 21 AMPs are best characterized and grouped into seven classes, including cecropins, drosocin, attacins, diptericins, defensin, drosomycins, and metchnikowin. Only six genes are present in the honey bee genome, such as abaecin, apidaecin and defensins. At least seven AMP families have been identified in the silkworm genome, of which only three are in common with those of *D. melanogaster* and *A. gambiae*. Genes encoding gloverin, moricin, enbocin, and lebocin exist in *B. mori* but not in *D. melanogaster*. In all the three shared AMP families, attacins, cecropins, and defensins are highly divergent: only one confident 1 : 1 : 1 ortholog (BmCecD2/DmAnp/AgCec4) exists. In the silkworm genome, about 39 AMPs were identified and are primarily comprised of cecropins, moricins and gloverins. Cecropins, attacins and defensins have been defined in many insects, whereas gloverins and moricins have so far been found only in Lepidoptera. Cecropins in the silkworm can be classified into five

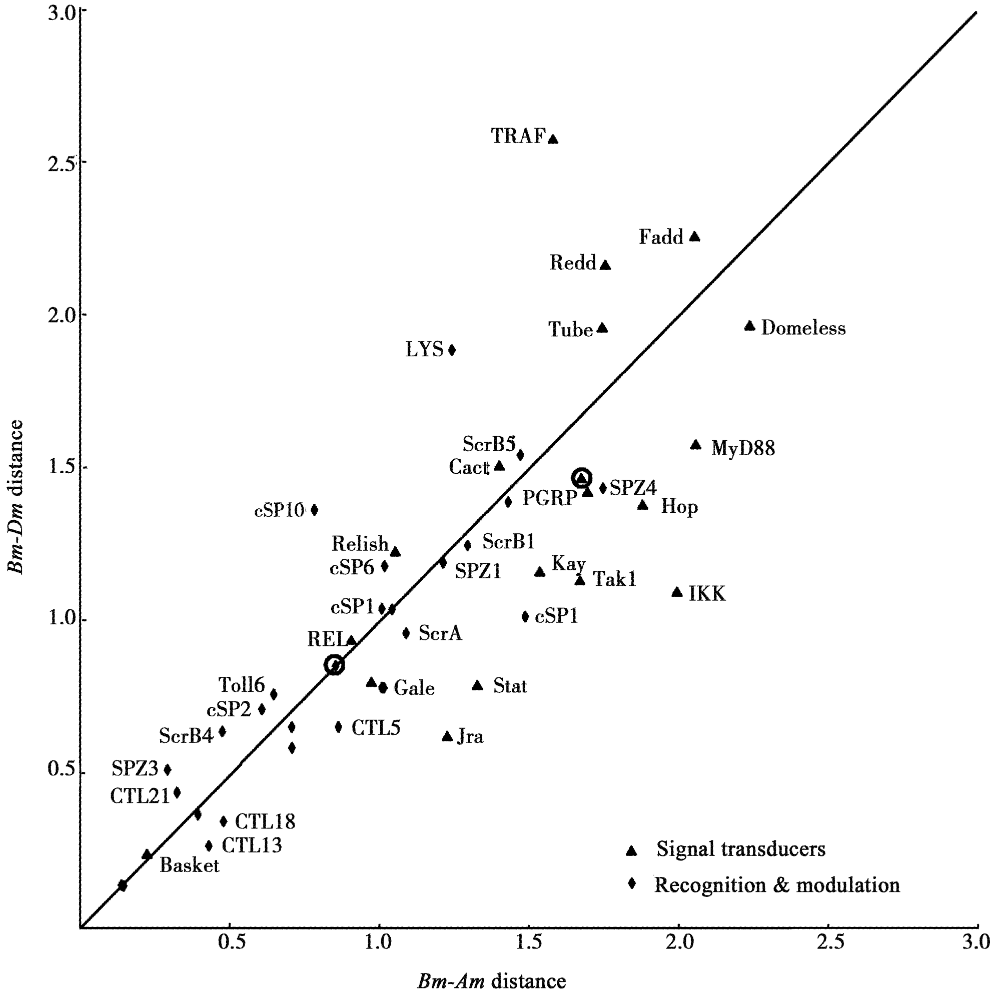

Fig. 2 Distance of orthologous genes

Signal transducers are indicated by triangles. Genes involved in recognition and modulation are indicated by diamonds. The rings indicate the average distance for signal transducers and recognition & modulation, respectively.

subtypes, from A to E, and effectively act on both bacteria and some fungi. The gloverin family includes seven members in the silkworm and the protein can be accumulated in the hemolymph of *Escherichia coli* immunized larvae. By using *in vitro* expression and antimicrobial activity assays, the members of gloverin family have been assigned distinctly different activities against different pathogens, including Gram-negative and Gram-positive bacteria, fungi and virus. The mature gloverins have also been isolated from lepidopteran insects *Hyalophora gloveri* and *Trichoplusiani*, and shown to markedly inhibit the growth of *E. coli* (Axen *et al.*, 1997; Lundstrom *et al.*, 2002).

With the exception of AMP families, three genes encoding PPOs were identified and are species-specific in the silkworm genome (Fig. 3: A). In *M. sexta*, two PPO and three PPO-activating proteases (PAPs) have been reported to function in extracellular serine proteinase (SP) cascade (Kanost *et al.*, 2004). PPOs require the extracellular SP cascade for the cleavage reaction. About 133 SPs were identified in the silkworm genome. Only 15 of these genes contain regulatory clip-domain, less than *D. melanogaster* and *A. gambiae*. However, due to the lack of confidence in the quality of certain silkworm SP pseudogene sequences, the gene family in this species might turn out to be somewhat larger than currently predicted. Inhibitors of SPs (SRPN) are present in the haemolymph and form complexes with diffusing proteinases to maintain homeostasis. There are 15 inhibitors of SPs in the silkworm genome, less than in *D. melanogaster* (30) or *T. castaneum* (31). This family appears specifically expanded and diversified under the selection pressure potentially imposed by the SP family duplication in *T. castaneum* (Zou *et al.*, 2007).

3.4 Signaling pathways for immune response

3.4.1

The Toll pathway: Toll receptors play

important roles in insects both in development and the establishment of embryonic dorsal-ventral polarity as well as innate-immune response against pathogens. In *D. melanogaster*, a cytokine ligand Spz binds to the extracellular domain of Toll and activates the Toll pathway to regulate the expression of *Drosomycin*. Six Spz-like molecules and nine Toll receptors are encoded in the *Drosophila* genome, respectively. In the silkworm genome, six Spz-like molecules were identified, four of which, including Spz1 and Spz3 – 5, have precise 1 ∶ 1 ∶ 1 ∶ 1 orthologous relationships with those in other insects examined (Fig. 3: B). When injected with BmSpz1, the transcripts of the silkworm AMP genes greatly increased. Similar results were found in *M. sexta* (Wang *et al*., 2007). The Toll family of *B. mori* is composed of 13 members (Cheng *et al*., 2007). *B. mori* Toll-1 expression was strongly down-regulated after injection with lipopolysaccharide (LPS), suggesting that the gene has a function similar to *Drosophila* Toll in response to LPS signaling (Imamura and Yamakawa, 2002). The Toll-6 and -8 have apparent 1∶1∶1∶1 orthologs in the four species. The Toll-9 seems to be absent in *A. mellifera* (Fig. 3: C). One homologue of ‘Dorsal’ coding two mRNAs named *BmRelA* and *BmRelB* through alternative splicing has been identified in the silkworm genome (Tanaka *et al*., 2005). Interestingly, the *BmRelA* and *BmRelB* strongly activated the Lebocin 4 and Attacin *in vitro*, respectively. Functional assays using BmRel indicate that the Rel homology domain can bind specifically to kappa B elements of AMP genes (Tanaka *et al*., 2005). The intracellular factors MyD88, Pelle, Tube, TRAF2 and cactin appear to be present in the silkworm genome as well as in *D. melanogaster* and *A. mellifera*. Candidate extracellular components for activation of the Toll pathway include Gram-negative binding protein (GNBP), PGRP, clip-serine proteases and serine protease inhibitors. The main effectors are comprised of AMPs and LYSs. Present evidence indicates that the inducible expression of AMP genes is involved in the Toll pathway as it is in *D. melanogaster*. The Toll pathway of insect species shares significant similarities with the vertebrate cascade of the TLRs and Interleukin-1, suggesting an evolutionarily conserved mechanism.

3.4.2 The Imd pathway: The Imd pathway is mainly responsible for immunity from Gram-negative bacterial infection via activation of PGRP-LC and mediates the induction of several AMP genes through the transcription factor Relish in *D. melanogaster*. The main components of this pathway have been identified by reverse genetic approaches and genetic screens, including FADD, Dredd, TAK1, TAB, IKKβ, IKKγ, and Relish. Except for IKKβ, the orthologous components of this pathway are present in the silkworm genome. One identical Relish ortholog is also evident (Fig. 3: D). The BmRelish gene also produces two cDNAs designated BmRelish-1 and -2. BmRel and BmRelish activate AMP genes *in vitro* (Tanaka *et al*., 2005; Tanaka *et al*., 2007). Compared with the genomes of other insect species, the Imd pathway is evolutionarily conserved. In *Drosophila*, the JNK pathway shares the up-stream components of the Imd pathway. Bacterial infection can activate this pathway, but the molecular mechanisms in the immune response are still unclear. The JNK components MKK7, JNK, c-jun, and Kay are highly conserved in the silkworm genome.

3.4.3 The JAK-STAT pathway: Like the Toll pathway, the JAK-STAT pathway also plays dual biological functions in development and immunity. This pathway was originally defined in the embryogenesis for somitic segmentation. The STAT protein is accumulated in the nucleus after immune challenge in the mosquito, first suggesting that the JAK-STAT pathway is involved in insect immunity (Barillas-Mury *et al*., 1999). This pathway is also involved in antiviral response in *Drosophila*. JAK/STAT-deficient flies are sensitive to infection with the *Drosophila* C virus (Dostert *et al*., 2005). Immune genes encoding complement-like thiolester-containing proteins and the stress genes *Turandot* are also regulated by the JAK-STAT pathway. The key ligand unpaired and effector Turandot appear to be absent in the silkworm genome and the honeybee genome as well. However, three main cellular components including cytokine receptor Domeless, janus kinase Hopscotch and the STAT transcription factor genes are present in the silkworm genome. The core members of JAK-STAT signaling pathway are significantly conserved, suggesting that an unknown ligand may trigger the JAK-STAT pathway in *B. mori* and *A. mellifera*. In addition to the core components, the genes for negative regulators including three suppressors of cytokine signaling (SOCSs) and one protein inhibitor of activated STAT (PIAS) were also identified in the silkworm genome.

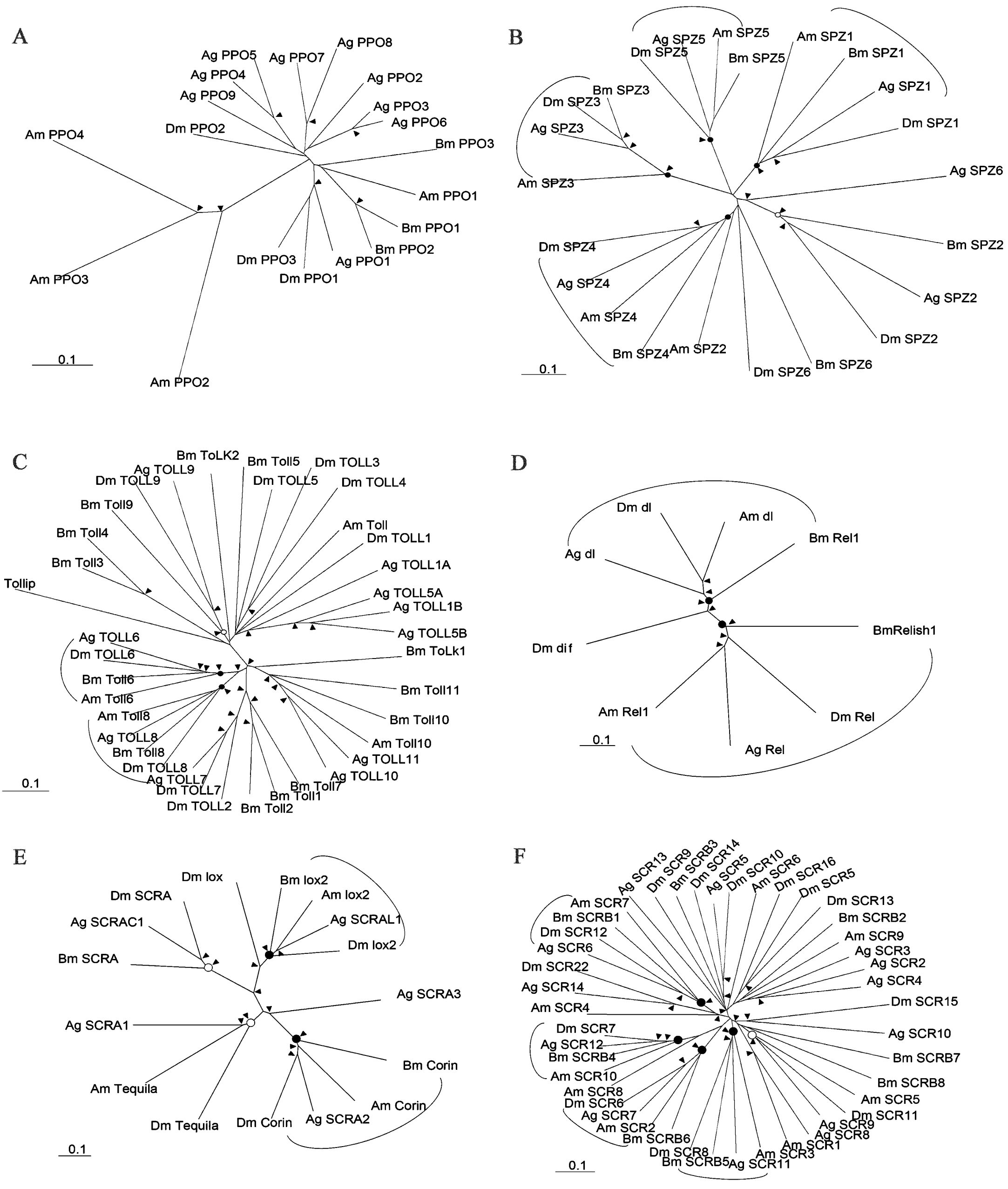

Fig. 3 Phylogenetic relationships of immune-related gene family members for *Bombyx mori* (*Bm*), *Drosphila melanogaster* (*Dm*), *Anopheles gambiae* (*Ag*) and *Apis mellifera* (*Am*)

A: PPO family; B: Spz family; C: Toll family; D: Rel family; E: SCRA family; F: SCRB family. Black arrowheads at nodes denote bootstrap values greater than 800 from 1 000 trials. The filled dots indicate 1:1:1:1 orthologs. The blank dots indicate 1:1:1 orthologs.

3.5 Pattern recognition receptors

Recognition of invading pathogens is the first step in insect defense response and is mediated by PRRs, primarily including PGRP, GNBP, scavenger receptors, C-lectins, and Galectins. There are 11 PGRPs in the silkworm genome, compared to 13 and 4 in *D. melanogaster* and *A. mellifera*, respectively. In *Drosophila*, PGRP-LCs mediate signals from Gram-negative peptidoglygans and activates the Imd pathway. Two families of PRRs were initially defined for their capacity to bind microbial ligands and to activate the prophenoloxidase cascade through a serine protease cascade (Ochiai and Ashida, 1988; Yoshida *et al.*, 1996). PGRPs were first purified from silkworm haemolymph and found to function as a PRR to recognize peptidoglycan and Gram-positive bacteria (Yoshida *et al.*, 1996). *B. mori* GNBP is constitutively expressed in the fat bodies and has strong affinity to the Gram-negative bacteria (Lee *et al.*, 1996), whereas the beta-1,3-glucan recognition protein (βGRP) has strong specific affinity to fungal cell wall components (Ochiai and Ashida, 1988). There are four GNBP genes in the silkworm genome, compared to 3 and 2 in *D. melanogaster* and *A.*

mellifera, respectively. GNBP1 and GNBP2 appear to have originated in the silkworm.

Cellular immune response including phagocytosis, encapsulation and nodulation and play crucial roles in the insect systemic immune defense (Lavine and Strand, 2002). In Lepidoptera, the plasmatocyte spreading peptide (PSP) belonging to the ENF-peptide family has been shown to be a plasmatocyte activator and to regulate plasmatocyte adhesion. There are two genes encoding paralytic peptides in the silkworm genome, which contain consensus N-terminal sequence (Glu-Asn-Phe) like that of PSP. Interestingly, eight genes encode paralytic peptide-binding proteins similar to the growth blocking peptide-binding protein of *Pseudaletia separata*. Recently, systems biology and dynamic evolutionary analysis in *Drosophila* have been made in the molecular dissection of these processes (Sackton *et al.*, 2007; Stuart *et al.*, 2007). The exocyst complex has an evolutionarily conserved role in phagocytosis. We found that eight components of this complex (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) are present in the silkworm genome. *Drosophila* phagocytosis has been proved to involve several kinds of receptor proteins, which include EGF-domain protein Eater, scavenger receptor family and IgSF-domain protein Dscam (Lemaitre and Hoffmann, 2007). These receptors might be involved in the exocytosis during early phagocytosis. As in *A. mellifera*, three Class A scavenger receptors (SCRAs) were identified in *B. mori* and were obvious orthologs (Fig. 3: E). BmSCRA contains a partial C-type lectin domain, whereas Bm_lox2 has the Lysyl oxidase (lox) domain of human Lox proteins. There are 8 Class B scavenger receptors (SCRBs) in *B. mori*, 4 of which show orthologous relationships (Fig. 3: F). Only one Class C scavenger receptor was found in *B. mori* and is orthologous with *Drosophila* SR-CI. In addition to SCR, three subclasses of *Drosophila* Nimrod are present in the silkworm genome and are well conserved.

3.6 Oxidative defense

The local generation of reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radical, is an important mechanism during immune response. However, they are potentially toxic to the host and cause oxidative damage to proteins, nucleic acids and lipids (Dalton *et al.*, 1999). To eliminate the damage caused by ROS, the hosts have evolved protective systems such as superoxide dismutases (SOD), catalases (CAT) and peroxidases (PX). In *B. mori*, the mRNA expression levels of thioredoxin (Trx) and thioredoxin peroxidase (Tpx) in the fat body were greatly increased when larvae were injected with microorganisms, suggesting that they play a protective role against oxidative stress caused by microbial infection (Lee *et al.*, 2005; Kim *et al.*, 2007). When compared with the *D. melanogaster*, *A. gambiae* and *A. mellifera* genomes, the silkworm genome has 6, 7 and 23 genes respectively, encoding SODs, CATs, and PXs. The PXs mainly include 17 heme-containing peroxidases (HPX), 4 thioredoxin peroxidase (Tpx), and 2 glutathione peroxidases (GPXs). The number of these genes is roughly similar to the other three insects, and most of them showed remarkable orthologous relationships.

4 DISCUSSION

As an important lepidopteran model, research on the immunity of the silkworm has been a focus from the last century. In this work, we identified 218 immune-related genes from 21 gene families based on the updated silkworm genome. These genes are associated with recognition, signaling modulation, effectors and oxidative defense. When compared with the *D. melanogaster*, *A. gambiae*, and *A. mellifera* genomes, the silkworm genome possesses a greater number of immune-related genes than that of the social insect, *A. mellifera* (Evans *et al.*, 2006). The immune-related genes of *B. mori* have the common hallmark of gene duplication similar in response to complicated evolutionary pressures in *Drosophila* and *Anopheles*, and most of the immune-related genes are significantly similar to *A. mellifera* in the sequence conservation, especially genes functioning in signaling transduction pathways. These components of signaling pathways demonstrate high degree of conservation in terms of phylogenetic orthologs within insect groups, but are in fact divergent at the amino acid sequence level. In contrast, molecules involved in recognition and modulation show higher conservation than signal transducers in spite of species-specific gene duplication of some families, but orthologs are markedly lacking. The phylogenetic analyses suggest that hosts readjust the defense strategies by gene duplication and sequence divergence of immune-related genes to protect them from pathogens.

Compared to other four insect species, the silkworm has some different gene families including recognition receptors and effectors. Species-specific gene families may reflect different physiology, feeding behaviour and ecology. For instance, the mosquito-specific duplication of FREPs, TEPs and PPOs might be partly linked to haematophagy (Waterhouse *et al.*, 2007). AgTEP1 belongs to the species-specific duplication and functions as recognition receptor to bind to pathogen surfaces and promote phagocytosis of blood cells and killing of *Plasmodium* parasites (Blandin *et al.*, 2004). The silkworm contains a remarkably high number of AMPs. Three gene families of cecropin, moricin and gloverin in the silkworm contain more members than those in other insects. Cao's group had tested their induced expression profile by real-time PCR and found a significant difference in their transcription levels after injection of different microbes (data unpublished). Furthermore, these AMPs have also been expressed *in vitro* and their antimicrobial activity was tested against more than ten kinds of microbes (unpublished). The results indicated that antimicrobial activity and spectrum of these AMPs were distinctly different, suggesting that the silkworm gene families of AMP have expanded and diversified under selective pressure. Interestingly, cSPs, LYS and galectins were found to be expanded only in Diptera (Waterhouse *et al.*, 2007). Presumably, Diptera-specific gene duplication is associated with their habitats. It is well known that both the silkworm and honeybee are phytophagous insects, the former is regarded as an oligophagous insect that feeds on mulberry leaves as its natural food; the latter has relatively simple food source, pollen and nectar.

Innate immunity is an evolutionarily conserved system. In insects, the response to pathogens is regulated by four signaling pathways, including the Toll, Imd, JNK, and JAK/STAT. Seventeen genes encoding components of these pathways have been identified in the silkworm genome, which exhibits remarkable orthologous relationships with *D. melanogaster*, *A. gambiae* and *A. mellifera*. Recent studies about expression regulation of AMPs have been reported in *B. mori*. Two NF-κB like transcription factors, BmRel and BmRelish, are capable of specific binding to different NF-κB elements in the upstream regions of AMP genes, suggesting that they regulate the expression of AMPs by a unique model (Tanaka *et al.*, 2005; Tanaka *et al.*, 2007). In addition, in *B. mori* Spatzle, an endogenous ligand protein of Toll receptors, was markedly induced after infection of *E. coli*. Overexpression of BmSpzl significantly induced the expression of AMPs (Wang *et al.*, 2007). The sequence conservation and experimental evidence suggest that the lepidopteran silkworm possesses the conserved Toll and Imd pathways for manipulating the expression of AMP genes in response to infection of bacteria and fungi.

Viral diseases are some of the most common infections in insects such as nuclear polyhedrosis, cytoplasmic polyhedrosis, infectious flacherie and densonucleosis virus. In the silkworm, three recessive genes (*nsd-1*, *nsd-2* and *nsd-Z*) and one dominant gene (*Nid-1*) have been defined and located for resistance to a densonucleosis virus (Abe *et al.*, 2000; Li *et al.*, 2006). In addition, several antiviral molecules have been reported. Three enzymes including a silkworm lipase, a serine protease and a soluble NADH-oxidoreductase-like protein have been isolated from the digestive juice that show strong antiviral activity against silkworm nucleopolyhedrovirus (Ponnuvel *et al.*, 2003; Nakazawa *et al.*, 2004; Selot *et al.*, 2007). Another lepidopteran-specific protein Hemolin, with four I-set type immunoglobulin domains, is induced and expressed after baculovirus and dsRNA introduction, suggesting that hemolin is used in antiviral defense (Hirai *et al.*, 2004). In *Drosophila*, the JAK/STAT pathway could be associated with regulating effectors for the rejection of the *Drosophila* C virus. Although information on gene expression regulation for viral infection and immune defense of hosts is scant, studies have suggested the presence of an antiviral immune system in the silkworm.

The wild silkworm, *Bombyx mandarina*, is the ancestor of the domesticated silkworm and subject to greater and more complex ecological pressures. On the basis of comparative analysis, studies on the immunity of wild and domesticated insects may reveal differential defense mechanisms elicited under selective pressures. The evolutionary modes will also assist in exploring the evolution of the innate immune mechanism in Lepidoptera. They will also contribute to the sericultural field by establishing transgenic non-susceptible strains of silkworm and to agriculture for better control of lepidopteran pests.

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家蚕免疫相关基因和信号途径的鉴定和比较分析

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摘要：家蚕 *Bombyx mori* 是一种重要的经济昆虫，在中国约有5 000年的驯化历史。家蚕分子免疫学方面的最新研究已经初步勾勒出其先天免疫的轮廓。本研究基于更新的家蚕基因组数据，通过与黑腹果蝇 *Drosophila melanogaster*、冈比亚按蚊 *Anopheles gambiae*、意大利蜜蜂 *Apis mellifera* 和赤拟谷盗 *Tribolium castaneum* 基因组的比较分析，鉴定了家蚕21个免疫相关基因家族的218个基因，其编码产物包括模式识别受体、信号传导因子、效应分子和氧化防御相关的酶类。尽管信号传导因子的序列分化较大，但系统进化分析显示它们在不同昆虫间呈明显的直系同源关系。相反，与识别、调制和效应因子相关的基因的序列保守性更高，但是这些基因家族明显缺乏直系同源基因，由此推测这些基因是由物种特异的基因复制机制产生的。结果提示家蚕拥有与其他昆虫相同的免疫应答调控的分子机制，而且家蚕同样可以通过基因复制及其序列分化等方式调节防御策略。

关键词：家蚕；先天免疫；抗菌肽；氧化防御；基因表达；信号途径

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